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SPONTANEOUS REACTIVATION OF ACETYLCHOLINESTERASE FOLLOWING ORGANOPHOSPHATE INHIBITION

II. CHARACTERIZATION OF THE REACTIVATING COMPONENTS

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Summary

Repeated cycles of inhibition by a variety of organophosphates followed by spontaneous reactivation reveal a component of electric eel acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) which preferentially reactivates. That the observed enzymatic activity truly resides in acetylcholinesterase is indicated by its sensitivity to a specific inhibitor and by molecular weights for subunits and native enzyme which are approximately the same as those for the major fraction of enzymatic activity which behaves in the classical manner. The K_m values for phenyl acetate of the two components are similar but the rate constant for covalent bond formation, k_2, with isopropyl *m*-nitrophenyl methylphosphonate is greatly reduced in the spontaneously reactivating species. The molecular basis for these observations is discussed.

Introduction

The existence of multiple molecular forms of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is well documented [1—3]. Some of these forms may be due to incomplete proteolysis [4] or dissociation into active dimers [5]. Certainly, unfractionated acetylcholinesterase is heterogeneous with respect to its sensitivity to organophosphate inhibition [6] and forms of acetylcholinesterase which differ in their electrophoretic mobility also display a range of sensitivities to organophosphate inhibition [7]. Therefore, it is reasonable to expect that different forms of acetylcholinesterase should spontaneously reactivate with different rates to various extents.

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Abbreviations: Sarin, isopropyl methylphosphonofluoridate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Soman, pinacolyl methylphosphonofluoridate; DFP, diisopropyl phosphonofluoridate; SDS, sodium dodecyl sulfate; BW 284C51, 1,5-bis-(*N*-allyl-*N*,*N*'-dimethyl-4-ammoniumphenyl)-petan-3-one dibromide, *m*-nitro Sarin, isopropyl *m*-nitrophenyl methylphosphonate.

The present study extends our previous observation [8] that, contrary to the statement of Hobbiger [9], acetylcholinesterase inhibited by Sarin at pH 7.60 is capable of spontaneous reactivation. We now inquire whether the enzyme activity which reappears after inhibition definitely resides in acetylcholinesterase, whether there are molecular species which preferentially reactivate, and whether other types of biochemical behavior correlate with the ability to reactivate spontaneously.

Materials and Methods

General. Acetylcholinesterase from *Electrophoresus electricus* was either purchased from Worthinton Biochemicals, Inc., or prepared in house. Preparations from both sources hydrolysed approx. 1000 μmol acetylcholine/min per mg when assayed at 25° C and pH 7.0. The enzyme was stored at a concentration of 6.7 mg/ml in 0.02% NaN_3, 0.225 M KCl and 0.1% gelatin.

The enzymatically active half-monomer of acetylcholinesterase (7.4 S acetylcholinesterase) was prepared as described by Millar et al. [5], with one exception. The sedimentation markers we used in the sucrose gradient were bovine liver catalase (11.3 S) and horseradish peroxidase (3.85 S). The addition of 2.0 μl of our 7.4 S acetylcholinesterase preparation to 1.0 ml of 0.10 M MOPS (pH 7.60) resulted in a change of 0.457 unit/min when assayed with phenyl acetate [8].

Repeated cycles of inhibition and reactivation. 1—5 μl of a concentrated aqueous stock solution of the inhibitor were combined with 1.2 mg of enzyme in 300 μl of buffer containing 0.033 M MOPS (pH 7.6) to give final inhibitor concentrations of 1.0, 3.8 and 6.8 μM for Soman, Sarin and DFP, respectively. Incubation was continued at room temperature until less than 0.1% of the initial activity was detectable, i.e. about 5 min for Soman or Sarin and 30 min for DFP. In order to eliminate excess inhibitor, the entire sample was immediately applied to the top of a 1.5 ml column of Sephadex G-75 (medium) packed in a Pasteur pipette and eluted with 0.10 M MOPS buffer. Fractions of 0.2 ml volume were collected and assayed for activity immediately and after incubation for 18 h at 25° C. Aliquots of the fractions containing peak activity, usually numbers 3—5, were then pooled and subjected to inhibition at the same concentration of organophosphate followed by gel filtration, incubation and assay as described above. Since the recovery from each cycle was virtually complete, the procedure could be repeated up to two additional times with retention of sufficient enzyme activity for accurate assays. In several experiments, uninhibited controls were subjected to three cycles of gel filtration, incubation and assay, followed by a final cycle which included initial inhibition. This protocol was designed to eliminate the possibility that the steps other than inhibition were responsible for generating a species which preferentially reactivated.

In the inhibition-spontaneous reactivation experiments using the half-monomer of acetylcholinesterase, 100 μl of 0.10 M MOPS buffer (pH 7.60) containing 0.01% bovine serum albumin was added to 200 μl of our 7.4 S acetylcholinesterase preparation. A control assay was run using 2.0 μl of this solution and the remaining 298 μl was inhibited with 1.0 μl of $5.6 \cdot 10^{-4}$ M

Soman in 0.9% saline solution. After 3 min, 10.0 μl of the inhibited enzyme solution was removed and assayed. It showed a residual activity of 0.08% of the control. The remaining 288 μl of inhibited enzyme solution was applied to the top of a 1.5 ml column of Sephadex G-75 (dry bead diameter of 40—120 μm) packed in a Pasteur pipette and eluted with MOPS buffer. Fractions of 0.2 ml were collected and assayed for activity after overnight incubation at 25° C. The spontaneous return of activity observed was 1.46% (i.e. 1.54% minus the 0.08% residual activity). Qualitatively similar results were obtained using a 7.4 S acetylcholinesterase sample of lower activity from the sucrose density gradient.

Determination of kinetic constants for phenyl acetate hydrolysis. Enzyme which had been subjected to three cycles of Soman inhibition and reactivation as described above was diluted in 1.0-ml cuvettes which contained 0.10 M MOPS (pH 7.60). After equilibration to 25° C, varying amounts of phenyl acetate were added from stock solutions of the ester in acetonitrile (spectro grade from Eastman Kodak Co.). Neat acetonitrile was added as needed to keep the volume added constant in each assay (10 μl per ml). Data from at least two rate determinations at each concentration of phenyl acetate was plotted in double reciprocal form and a straight line was fitted by a weighted least squares formula [10]. Constants for phenyl acetate hydrolysis were determined over the same substrate concentration range and under exactly the same conditions with enzyme which had never been inhibited.

Kinetics of inhibition of reactivated acetylcholinesterase by m-nitro Sarin. We employed the method of Hart and O'Brien [11]. Their expression for the phosphorylation rate constant, k_2, can be simplified to:

$$k_2 = \frac{\Delta \ln v}{\Delta t} \cdot \frac{1}{1 - \frac{v_0}{v_c}} \tag{1}$$

where v is activity remaining at time t, v_c is the rate of substrate hydrolysis in the absence of inhibitor and v_0 is the initial rate of substrate hydrolysis in the presence of inhibitor. This expression is easier than that of Hart and O'Brien to manipulate and makes it clear that measurement of the affinity of the enzyme for substrate and inhibitor are not required in the calculation of k_2. Their expression for inhibitor dissociation constant, K_d, was used without modification.

$$K_d = \frac{K_m[PX]}{(K_m + [S])\,(v_c/v_0 - 1)} \tag{2}$$

K_m is the Michaelis constant for substrate, [PX] is the inhibitor concentration and [S] is the initial substrate concentration.

The inhibition experiments themselves were performed at 25° C with the concentrations of buffer and phenyl acetate used in the standard assay system described above. Control acetylcholinesterase or enzyme reactivated after three cycles of Soman inhibition was introduced into a 1.0 ml assay system at an activity of about 0.073 absorbance unit per min. A 2.0 μl aliquot of *m*-nitro-Sarin in acetonitrile was added shortly thereafter giving a final inhibitor concentration of $8.82 \cdot 10^{-5}$ M and the rate of substrate hydrolysis was followed for 15—20 min. The enzyme activity remaining after appropriate time intervals

was determined by manually constructing tangents to the absorbance vs. time curve.

Molecular weight of acetylcholinesterase subunits. Uninhibited enzyme or material which had been subjected to three cycles of DFP inhibition and reactivation was exposed to 5 μM [^{3}H]DFP (specific activity 880 Ci/M, New England Nuclear) in 0.033 M MOPS (pH 7.6) for 30 min at room temperature. The samples were then passed through small columns of Sephadex G-75 in the usual manner. Fractions (0.2 ml) were collected directly into tubes containing 20 μl of 10% (w/v) SDS. Radioactivity in an aliquot of each fraction was measured in a Packard scintillation counter after dispersion in toluene-based phosphor containing 20% (v/v) Triton X-100 (Rohm and Haas). Fractions from the void volume were pooled and an aliquot subjected to electrophoresis in 6% polyacrylamide gel in the presence of SDS and β-mercaptoethanol [12]. Polypeptides of known molecular weight (human transferrin, bovine serum albumin, carboxypeptidase, trypsin and ribonuclease) were simultaneously electrophoresed in parallel gels. All gels were stained with 0.1% Coomassie Brilliant Blue in 50% methanol/7% acetic acid mixture. Measurements for standard curve construction and determination of unknown molecular weights were made either by hand or from records made using the Gilford linear transport apparatus (Gilford Instrument Laboratories, Inc. Oberlin, Ohio).

The locations of stained bands were noted. Then gels containing ^{3}H-labeled proteins were sliced and radioactivity determined in a Packard scintillation counter after incubation of each slice in 0.2 ml of NCS tissue solubilizer (American/Searle Corp.) for 2 h and addition of a toluene-based phosphor. Preliminary experiments showed that recovery of radioactivity was identical whether gel slices were stained and subjected to this brief alkaline hydrolysis or completely combusted with collection of ^3H_2O.

Molecular weights of native and spontaneously reactivated acetylcholinesterase. Samples of up to 100 μl total volume of uninhibited enzyme or material which had been subjected to three cycles of DFP inhibition and reactivation were applied to 2.5—27% Gradipore survey gels (Isolab, Inc. Akron, Ohio) and electrophoresed at 4 mA per tube for 20 h at 0.4° C; longer running times resulted in no further migration. The electrophoresis buffer was pH 8.3 and contained 0.6 g trishydroxymethylaminomethane and 2.88 g glycine per l [13]. Constant recirculation of the buffer was required. Bands containing acetylcholinesterase activity were located by a staining method which uses acetylthiocholine as substrate [14]. It was absolutely necessary to rinse the gels in Tris/glycine buffer for 10—15 s to prevent artifactual staining by enzyme which was streaked along the surface of the gels while they were being removed from the tubes. The stained gels were scanned at 412 nm and then stored in 7% acetic acid. In control experiments, 0.2 μM BW 284C51 (Burroughs Wellcome) was incorporated into the staining mixture.

Standards of known molecular weight were made by using 8% formaldehyde at 25° C to produce random intermolecular cross-links in a 1 mg/ml solution of bovine serum albumin. Following incubation for 1 h, this mixture could be stored at 0° C indefinitely. After gradient gel electrophoresis, staining and scanning allowed us to visualize monomers and polymers up to hexamer and, occasionally heptamers, i.e. polymers of up to 408 000 and 476 000 molecular

weight. Plots of migration distance from the bottom of the gel versus logarithm of the calculated molecular weight were linear.

Results

Repeated cycles of inhibition and reactivation

The extent of recovery of enzymatic activity following repeated inhibition with DFP, Sarin or Soman is shown in Table I. Activity is expressed as percentage reactivation for a particular cycle. The behavior of the enzyme is qualitatively similar following inhibition with all three agents in spite of considerable differences in their structures. Therefore, the possibility that a minor contaminant in one of the inhibitor preparations or the optical isomers known to be present in Soman and Sarin is responsible for the spontaneous reactivation phenomenon can be discarded. The quantitatively different extents of reactivation observed with the three inhibitors are not surprising since the rates of reactivation of such closely related adducts as dimethyl- and diethylphosphoryl-acetylcholinesterase differ by a factor of 20 [16].

The increasing extent of reactivation upon repeated inhibition was reproducible, both with different batches of enzyme and after long intervals between experiments. We eliminated the possibility that gel filtration and incubation were encouraging the production of a modified enzyme by subjecting controls to three cycles of these procedures without exposure to inhibitor followed by inhibition as usual with DFP. The resulting reactivation was less that 1%, as compared with the value of 57% (Table I) expected if a rapidly reactivating species were being produced artifactually. Neither was it possible that incomplete reactivation after the first inhibition was responsible for spuriously increased extents of reactivation in subsequent cycles. The $t_{1/2}$ of 154 min previously observed [8] for spontaneous reactivation at pH 7.6 requires that after the 18 h incubation period used in the present experiments, no more than 1 part in 15 of the activity appearing after the second cycle of inhibition and reactivation could have been spuriously carried over from the first cycle.

These observations seemed to be compatible with two models: (1) the experimental manipulations give rise to a modified acetylcholinesterase or (2) the enzyme preparations are initially heterogeneous. Both explanations

TABLE I

PERCENT SPONTANEOUS REACTIVATION OF ACETYLCHOLINESTERASE FOLLOWING INHIBITION BY VARIOUS ORGANOPHOSPHATES

Spontaneous reactivation relative to activity remaining at the start of each inhibition cycle.

Inhibition cycle	Inhibitor					
	DFP		Soman		Sarin	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
1	0.08	0.11	0.66	0.79	0.72	1.18
2	28.2	28.1	34.3	33.3	11.6	15.0
3	—	57.0	68.1	69.1	—	49.3

require a two-component system consisting of one species, acetylcholinesterase (I), which reactivates slowly, if at all, with concurrent aging, and another species, acetylcholinesterase (II), which reactivates at least as rapidly as it ages. That is, acetylcholinesterase (I) behaves as classically expected, while acetylcholinesterase (II) comes to predominate upon repeated cycles of reactivation.

Determination of kinetic constants for phenyl acetate hydrolysis

The data plotted in Fig. 1 was used to obtain Michaelis constants of 1.32 mM (95% confidence limit, 0.45—2.18 mM) for reactivated (acetylcholinesterase (II)) and 2.21 mM (95% confidence limit, 1.23—3.20 mM) for control (acetylcholinesterase (I)) enzyme preparations. These values do not differ significantly since their 95% confidence limits overlap by a wide margin. It is only by coincidence that the lines of best fit appear to be parallel. At a substrate concentration of 3.68 mM both preparations were inhibited 98.6% by 4 mM BW 284C51, further evidence that acetylcholinesterase (II) is an acetylcholinesterase [17].

Kinetics of inhibition of reactivated acetylcholinesterase by m-nitro Sarin

The use of an inhibitor with *m*-nitrophenol as the leaving group instead of fluorine was dictated by preliminary experiments in which the reactions with

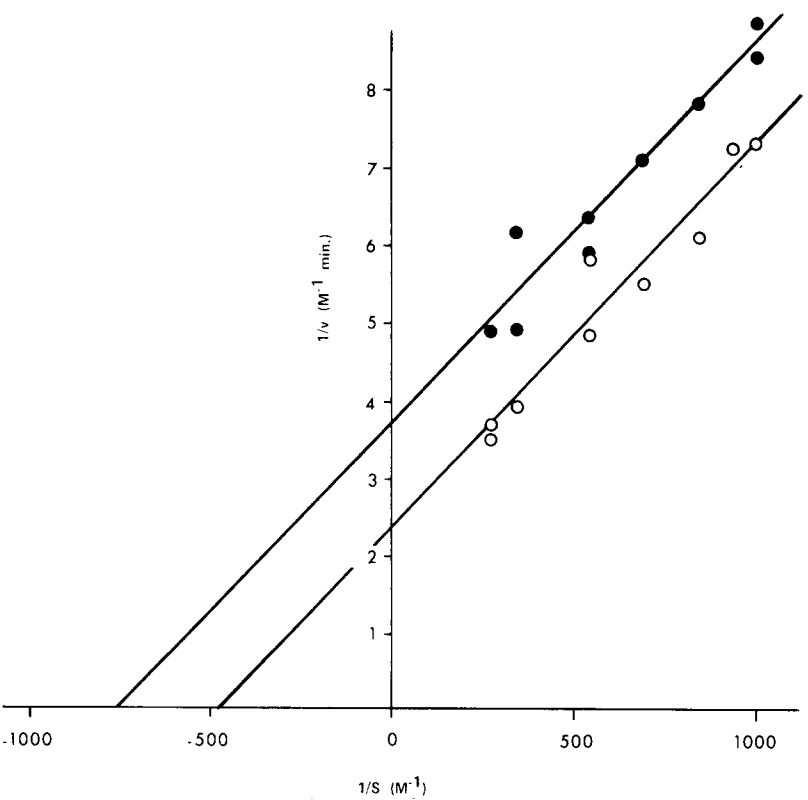

Fig. 1. Double reciprocal plots of rate of phenyl acetate hydrolysis by reactivated (●———●) and control (○———○) acetylcholinesterase preparations.

Sarin or Soman were too fast to obtain good kinetics. In Fig. 2, ln % of initial activity instead of ln v is plotted against time so that the inhibition of acetylcholinesterase (I) and acetylcholinesterase (II) can be compared more easily. It is evident that acetylcholinesterase (I) is much more sensitive to inhibition than acetylcholinesterase (II).

Extraction of K_d (dissociation constant) and k_2 (phosphorylation constant) from this data and from similar data for a preparation of active acetylcholinesterase dimers (7.4 S) is shown in Table II. K_d and k_2 for inhibition of the dimers and acetylcholinesterase (I) are quite similar, suggesting that the state of subunit aggregation has little effect on the inhibition reaction. The affinity of the acetylcholinesterase (II) for *m*-nitro Sarin is only moderately more than that of the controls while the phosphorylation constant for covalent bond formation is reduced by a factor of 7. Likewise, it should be noted that the bimolecular constant k_i, which is k_2/K_d, is affected by a factor of about 3. Failure to inhibit acetylcholineseterase (II) cannot have influenced the spontaneous reactivation experiments described above since the preparations were always assayed for complete inhibition prior to removal of excess inhibitor by gel filtration.

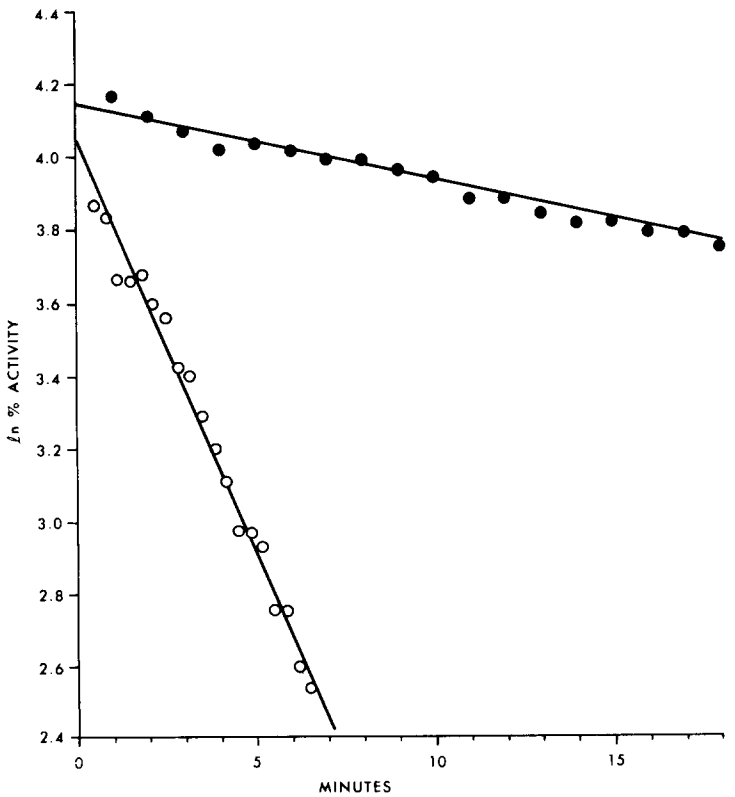

Fig. 2. Kinetics of inhibition of reactivated (●———●) and control (○———○) acetylcholinesterase preparations by *m*-nitro Sarin. (See Materials and Methods).

TABLE II

KINETIC CONSTANTS FOR INHIBITION OF ACETYLCHOLINESTERASE PREPARATIONS BY *m*-NITRO SARIN

	Control	Reactivated	Control dimers
$K_d(M \times 10^4)$	2.18 (2.00—2.52)	0.91	2.20
$k_2(min^{-1})$	0.598 (0.422—0.768)	0.082	0.546
$k_i(M^{-1} \times min^{-1})$	2743 (2080—3520)	901	2482

Molecular weights of acetylcholinesterase subunits

Polyacrylamide gel electrophoresis of [^{3}H]DFP-labeled acetylcholinesterase (I) and acetylcholinesterase (II) in the presence of 0.1% SDS yielded the distributions of radioactivity shown in Fig. 3. We do not consider the molecular weights of 58 500 and 62 500 for acetylcholinesterase (II) and acetylcholinesterase (I), respectively, to be significantly different, since the error in molecular weight obtained when samples are electrophoresed in separate gels is on the order of 5—10% [12,18,19]. Furthermore, the peak of radioactivity due to [^{3}H]DFP bound to acetylcholinesterase (II) corresponded exactly to the position of the stained band of acetylcholinesterase (I) which was present in the same gel.

Molecular weights of native and spontaneously reactivated acetylcholinesterase

Fractionation on polyacrylamide gradient gels of control acetylcholinesterase (I) which had been subjected to three cycles of chromatography and incubation revealed the components shown in Fig. 4. There is one major component (peak

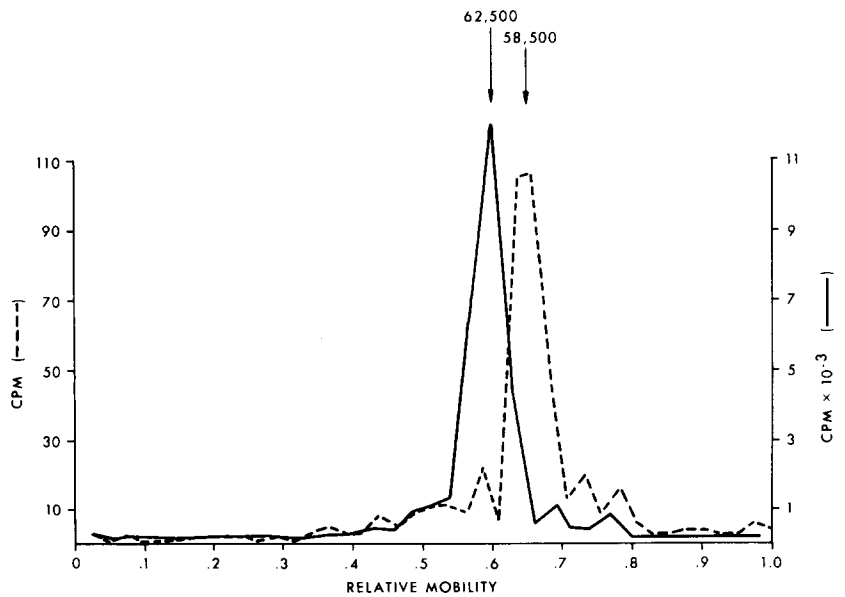

Fig. 3. Polyacrylamide gel electrophoresis in the presence of 0.1% SDS of reactivated (- - - - -) and control (———) acetylcholinesterase labeled with [^{3}H]DFP.

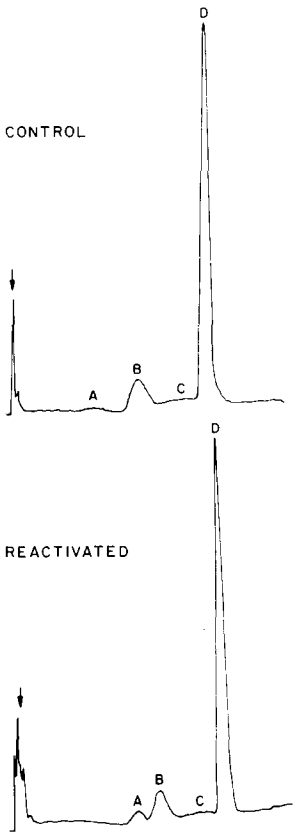

Fig. 4. Distribution of enzymatic activity following fractionation of control and reactivated acetylcholinesterase preparations on polyacrylamide gradient gels. Arrows indicate bottom (high polyacrylamide concentration) of the gels.

D) and one definite minor component (peak B) with small amounts of activity migrating both between these peaks and in advance of peak B. The approximate molecular weights of these components are given in Table III. Since the error in molecular weight determined by this method is probably quite large, the data are compatible with peak D being composed of tetramers of the

TABLE III

PROPORTION OF ACETYLCHOLINESTERASE ACTIVITY PRESENT IN VARIOUS MOLECULAR WEIGHT COMPONENTS

Activity peak *	Control		Reactivated	
	Molecular weight	Percentage of total activity	Molecular weight	Percentage of total activity
A	—	1.4	69 000	4.3
B	100 000	12.0	108 000	11.7
C	—	8.0	—	8.0
D	230 000	78.5	232 000	75.9

* Designations are the same as in Fig. 4.

60 000 molecular weight subunit found under denaturing conditions in the presence of SDS. Similarly, peak B would consist of dimers of the same sort. Assuming that the turnover numbers are the same for each species, the mass ratio of tetrameters to dimers calculated from their relative peak area is 6.5 to 1.

The molecular weight components obtained upon fractionation of acetylcholinesterase (II) obtained by three cycles of DFP inhibition and reactivation are basically similar to those in the control. Peak A is large enough to estimate its molecular weight at 69 000, corresponding to that expected for the single acetylcholinesterase subunit. All of the species present in the control and reactivated preparations are inhibited when BW 284C51 is included in the staining reaction mixture, thereby confirming the identity of the minor components as acetylcholinesterase [17].

Discussion

Two novel findings emerge from this study. First, reaction of electric eel acetylcholinesterase at pH 7.60 with three "irreversible" organophosphate inhibitors yields a minor phosphorylated acetylcholinesterase species that is capable of reactivating spontaneously. We have termed the major, slowly reactivating enzyme, acetylcholinesterase (I) and the minor, rapidly reactivating enzyme, acetylcholinesterase (II). Second, the observation that up to nearly 70% of the acetylcholinesterase (II) reactivates after repeated cycles of inhibition indicates that it must age much more slowly than acetylcholinesterase (I).

It has not yet been determined unequivocally whether acetylcholinesterase (II) is present prior to inhibition by organophosphates or is produced by a secondary reaction involving the inhibitor. The former explanation is consistent with the various organophosphates giving similar extents of reactivation after repeated cycles of inhibition. Despite the quantitative differences discussed above, definite and increasing reactivation after DFP inhibition indicates that reaction with stereoisomers and impurities could not be solely responsible for the reactivation phenomenon.

While there is abundant evidence that eel acetylcholinesterase is physically and chemically heterogeneous [2,4,7,19], the relationship of these data to the present findings is not clear. Since the 11 S and 7.4 S acetylcholinesterase are not interconvertible [5], the analogous spontaneous reactivation observed when these forms are inhibited with Soman and the similar molecular weight distributions of the native acetylcholinesterase (I) and acetylcholinesterase (II) (Fig. 4 and Table III) indicate that a particular state of subunit aggregation is not required for spontaneous reactivation to occur.

Previous analyses of the subunit composition of eel acetylcholinesterase [4,19—21] have indicated that, upon storage, proteolytic cleavage of the native enzyme subunits produces two major peptides, one of 20 000—25 000 molecular weight, and the other estimated to be of 50 000—59 000 molecular weight [4,20]. The enzyme used in this study was probably extensively proteolysed since we repeatedly found no evidence of a 70 000—80 000 molecular weight subunit either by [^{3}H]DFP labeling or by direct protein staining. Such proteolysis of the enzyme is probably irrelevant to spontaneous reactivation

since the reactivating species, acetylcholinesterase (II), is obviously not a minor component composed of undegraded subunits (Fig. 4, Table III).

The kinetics of inhibition by *m*-nitro Sarin may give a clue to a better understanding of the spontaneous reactivation of acetylcholinesterase (II). While the affinity of acetylcholinesterase (II) for this inhibitor is somewhat greater than that of acetylcholinesterase (I), the rate constant for covalent bond formation (k_2) for the former is reduced approx. 7-fold (Table II). Since experiments in progress (Lanks, K.W. and Lieske, C.N., unpublished data) indicate that the activation energy for reactivation of DFP-inhibited acetylcholinesterase (II) is similar to that observed for the spontaneous reactivation of erythrocyte acetylcholinesterase inhibited with dimethyl *p*-nitrophenyl phosphate [22], the spontaneous reactivation of acetylcholinesterase (II) appears to be a typical dephosphorylation process. Therefore, the spontaneous reactivation of acetylcholinesterase (II) may result from a distortion of the active site which decreases the rate of covalent bond formation and aging while increasing the rate of spontaneous reactivation and leaving the affinities for substrate or inhibitors relatively unaffected. The report that allosteric effectors can influence the aging rate of phosphorylated acetylcholinesterase [23] indicates that such a distortion can actually occur.

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